Emissive Synthetic Cofactors: Enzymatic Interconversions of tzA Analogues of ATP, NAD+, NADH, NADP+, and NADPH

Emissive synthetic cofactors: enzymatic interconversions of ^{tz}A analogues of ATP, NAD$^+$, NADH, NADP$^+$ and NADPH

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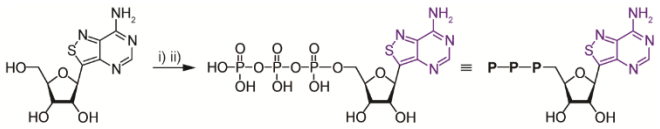
Abstract: A series of enzymatic transformations, which generate visibly emissive isofunctional cofactors based on an isothiazolo[4,3-*d*]pyrimidine analogue of adenosine (**^{tz}A**), is described. Nicotinamide adenylyl transferase condenses nicotinamide mononucleotide and **^{tz}ATP** to yield **N^{tz}AD$^+$**, which can be enzymatically phosphorylated by NAD kinase and ATP or **^{tz}ATP** to the corresponding **N^{tz}ADP$^+$**. The latter can be engaged in NADP-specific coupled enzymatic transformations involving conversion to **N^{tz}ADPH** by glucose-6-phosphate dehydrogenase and reoxidation to **N^{tz}ADP$^+$** by glutathione reductase. The **N^{tz}ADP$^+$/N^{tz}ADPH** cycle can be monitored in real time by emission spectroscopy.

Nucleic acids and their building blocks play central roles in all cellular events, including storage, retrieval, regulation and expression of genetic information, as well as signalling and metabolic pathways. This extensive biology necessitates the development of new tools for studying their recognition properties as well as alteration by endogenous and exogenous agents.[1] Emissive nucleoside analogues, when judiciously fabricated and incorporated, could serve as effective photophysical and mechanistic probes.[2] Indeed, extensive efforts have been dedicated to the development and refinement of such tools in recent years.[3–5]

Our focus over the past decade has been to design, synthesize, and implement new emissive nucleoside analogues that can faithfully replace their natural counterparts.[3,6] A guiding principle for the successful advancement of such probes is to curtail structural and functional perturbations (features defined as isomorphicity and isofunctionality, respectively), while endowing them with useful photophysical attributes. Several families of emissive analogs have been made and employed,[6] with the two emissive RNA alphabets based on the thiopheno[3,4-*d*]pyrimidine (**thN**)[7] and the isothiazolo[4,3-*d*]pyrimidine-cores (**^{tz}N**)[8] being most notable.[9–11]

While the incorporation of a single modified nucleoside into an oligonucleotide could be structurally and functionally benign, as detrimental effects might be masked, the adequate performance of nucleosides and nucleotides as cofactors and secondary messengers represents a much more demanding test for their isomorphism and isofunctionality. Along these lines we have initiated the exploration of synthetic emissive cofactors and their metabolic interconversions by transferases, kinases and hydrolases, with the goal of defining their biological recognition space and potential utility for investigating signalling and metabolic pathways.[12,13] In this contribution, we illustrate the enzymatic synthesis of the emissive and isofunctional **N^{tz}AD$^+$** from nicotinamide mononucleotide and **^{tz}ATP**. We then show that **N^{tz}AD$^+$** can be enzymatically phosphorylated by NAD kinase to the corresponding **N^{tz}ADP$^+$**, which in turn can be engaged in NADP-specific coupled enzymatic transformations involving conversion to **N^{tz}ADPH** by glucose-6-phosphate dehydrogenase and oxidation back to **N^{tz}ADP$^+$** by glutathione reductase. Rewardingly, the **N^{tz}ADP$^+$/N^{tz}ADPH** cycle can be monitored in real time by emission spectroscopy.



Scheme 1: Chemical synthesis of ^{tz}ATP. i) $POCl_3$, TMPP, 0°C to rt, 2 h; ii) tributylammonium pyrophosphate, TBA, 0°C to rt, 2 h, 17%.

A key precursor for the enzymatic fabrication of emissive adenosine-containing cofactors is isothiazolo-adenosine triphosphate (**^{tz}ATP**), which was synthesized from the corresponding nucleoside according to common protocols (Scheme 1).[14] To evaluate the biocompatibility of **^{tz}A**-based secondary messengers/cofactors, a multienzymatic assay was used in which a mixture of nicotinamide mononucleotide and **^{tz}ATP** is treated with nicotinamide adenylyl transferase 1 (NMNAT-1) and subsequently with *S. cerevisiae* alcohol dehydrogenase (ADH). The former enzyme catalyses the transfer of nicotinamide mononucleotide to ATP,[15] releasing inorganic pyrophosphate, while the latter oxidizes ethanol to acetaldehyde in presence of NAD$^+$ as a cofactor. Thus, to enzymatically access **N^{tz}AD$^+$**, a solution of **^{tz}ATP** and nicotinamide mononucleotide (NMN) in Tris buffer (pH 7.8) was treated with a recombinant human NMNAT-1 at 37°C. The use of an inorganic pyrophosphatase was found to be essential to avoid product inhibition of the transferase[16] and facilitate a quantitative reaction (conversion > 95%). This enzymatic transformation was followed in real time by monitoring the emission intensity at 410 nm (λ_{ex}: 330 nm) (Figure 1b). The conversion of **^{tz}ATP** into **N^{tz}AD$^+$** was depicted by a significant decrease in emission intensity over a 30 minutes window. The *in situ* formed **N^{tz}AD$^+$** was then converted to the corresponding **N^{tz}ADH** over 10 minutes by the addition of ADH and ethanol to the NMNAT-1-mediated reaction mixture. In a similar fashion to the previous enzymatic reaction, the reduction

of **N^{tz}AD$^+$** by ADH was characterized by a further decrease in emission intensity comparable to the one observed for the enzymatic conversion of **^{tz}ATP** into **N^{tz}AD$^+$** (Figure 1b). Final addition of excess acetaldehyde triggered a nearly instantaneous enzymatic oxidation of **N^{tz}ADH**, recovering 80% of the original emission signal of **N^{tz}AD$^+$**.

Steady state absorption and emission spectra recorded at the end (plateau) of each enzymatic transformation show that the conversion of **^{tz}ATP** to **N^{tz}AD$^+$** has no or little effect on the absorption spectra of the **^{tz}A** chromophore but does lead to diminished fluorescence (Figure 1c). Further emission quenching is seen upon reduction of **N^{tz}AD$^+$** to **N^{tz}ADH**, along with an increase in the optical density at 333 nm, consistent with a reduced nicotinamide moiety.[17] These observations reveal the high photophysical responsiveness of the emissive **^{tz}A** based cofactors. As a control, the same enzymatic cycle was performed with **^{tz}ATP** as the substrate, but without NMNAT-1. This reaction was monitored in real-time by ground state absorption and steady state fluorescence spectroscopies, showing little to no change in each corresponding spectra, thus indicating no conversion of **^{tz}ATP** over time (Figure S1).[14]

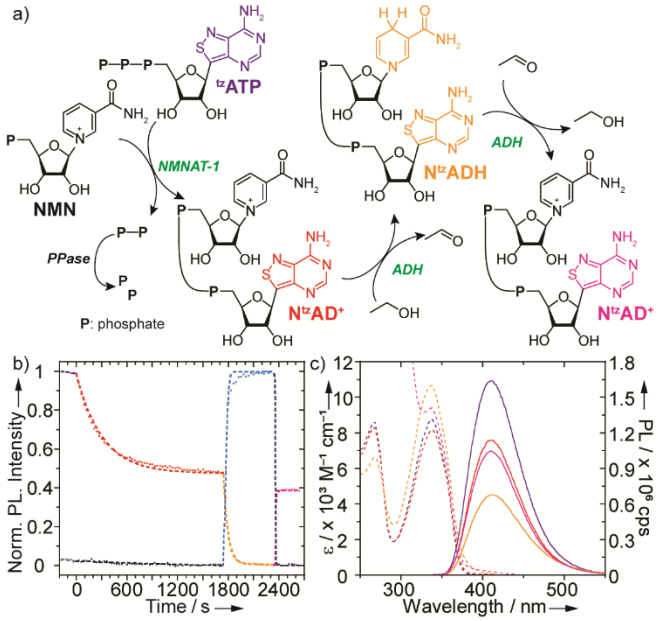


Figure 1: a) Enzymatic cycle for **N^{tz}AD$^+$** generation, consumption and regeneration with NMNAT-1 and ADH. b) Enzymatic transfer of NMN to **^{tz}ATP** (red) or ATP (black) by NMNAT-1 to form **N^{tz}AD$^+$** or NAD$^+$, followed by oxidation of ethanol to acetaldehyde with ADH, generating **N^{tz}ADH** (orange) and NADH (cyan), succeeded by a reverse enzymatic reaction with an excess of acetaldehyde to regenerate **N^{tz}AD$^+$** (pink) and NAD$^+$ (blue), monitored by real-time emission at 410 nm (λ_{ex}: 330 nm) and 465 nm (λ_{ex}: 335 nm) for the **^{tz}A**-based and native derivatives, respectively. c) Steady state absorption (dashed line) and emission (solid line) spectra of **^{tz}ATP** (purple), in situ generated **N^{tz}AD$^+$** (red), **N^{tz}ADH** (orange), and regenerated **N^{tz}AD$^+$** (pink), taken at the end of each time-based enzymatic reaction.

While numerous cellular processes utilize the NAD$^+$/NADH couple, several metabolic reactions exclusively use the NADP$^+$/NADPH pair, the corresponding monophosphorylated variant.[18] Natively, NAD kinase catalyses the transfer of a phosphate group from ATP to the 2' hydroxyl group on the adenosine's D-ribose moiety of NAD$^+$, while certain bacterial NAD kinases can also use polyphosphate minerals as a phosphate source.[19] To assess the enzymatic syntheses of **N^{tz}ADP$^+$** and **N^{tz}ADPH**, **N^{tz}AD$^+$** (chemically synthesized as recently described)[12b] was treated with *B. subtilis* NAD kinase (NADK) and ATP.[20] While the phosphorylation reaction was observed (Figure 2), buildup of the newly synthesized **N^{tz}ADP$^+$** resulted in product inhibition, as expected based on previous reports.[19b] To circumvent this issue, the kinase-mediated reaction was coupled to a second enzymatic system, utilizing the *in situ*-generated phosphorylated cofactor. Thus excess *S. cerevisiae* glucose-6-phosphate dehydrogenase (G6PDH), an enzyme responsible for the oxidation of glucose-6-phosphate (G6P),[21] was added to the reaction mixture. Upon coupling this enzyme to our process, the newly-formed **N^{tz}ADP$^+$** was indeed actively converted to **N^{tz}ADPH** via G6PDH, as illustrated by HPLC and HR-MS (See Figures 2d, S5 and S6). Importantly, NAD$^+$ kinase can also utilize **^{tz}ATP** as the phosphate donor, thus illustrating a native enzyme-mediated reaction, which utilizes two distinct synthetic cofactors (Figure S4).[14]

The NADK-mediated phosphorylation of **N^{tz}AD$^+$** to **N^{tz}ADP$^+$** is, as predictable, photophysically "silent", while the following reduction of the *in situ* formed **N^{tz}ADP$^+$** to **N^{tz}ADPH** via G6PDH shows significant photophysical changes, comparable to those seen for **N^{tz}AD$^+$/N^{tz}ADH** (Figure 2a). Thus, over 3 hours, an increase in absorbance at 333 nm and a concomitant decrease of the emission intensity at 410 nm (λ_{ex}: 330 nm) are observed (Figure 2b). The overall reaction half-time, derived from the

emission spectra variation over time (Figure 2c), are 3.3 and 3.6 × 10^2 s for the native and the ^{tz}**A**-based analogue nucleosides, respectively, assuming pseudo first-order kinetics.[14] This process was also monitored in parallel by HPLC, showing 82% conversion for **N^{tz}AD$^+$**, compared to the 90% conversion when using native NAD$^+$ (Figure 2d). Control experiments performed with **N^{tz}AD$^+$** in the absence of NADK yielded little to no variation in both the absorption and emission spectra (Figure S3).[14]

To assess the reverse reaction, **N^{tz}ADPH** was then subjected to treatment with *S. cerevisiae* glutathione reductase (GR). This enzyme converts oxidized glutathione (GSSG) to its reduced form (GSH) using NADPH as a cofactor, subsequently reducing it to NADP$^+$.[22] As seen in Figure 2b, this enzyme was able to instantly reduce GSSG to GSH with **N^{tz}ADPH**. Indeed, an instant restoration of visible fluorescence (λ_{ex} 330 nm, λ_{em} 410 nm; Figure 2b) was observed, demonstrating again the utility of our emissive analogues, where such enzymatic processes can be monitored by unique absorption and emission signal changes.

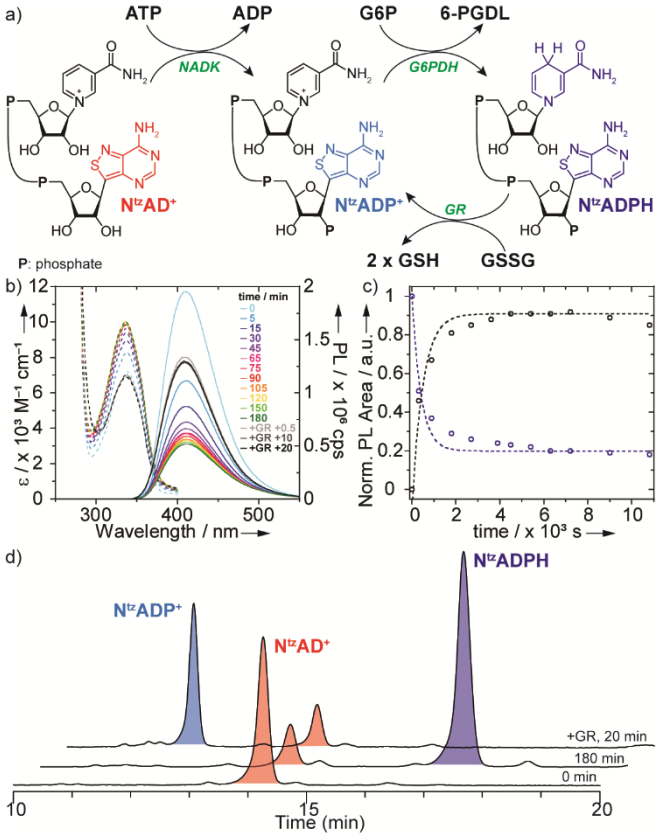


Figure 2: a) Enzymatic cycle for **N^{tz}AD$^+$** phosphorylation by NADK followed by G6PDH-mediated reduction of **N^{tz}ADP$^+$** to **N^{tz}ADPH** and final reoxidation of the latter by GR with GSSG as substrate. b) Time-dependent steady-state absorption and emission spectra for the enzymatic conversion of **N^{tz}AD$^+$** to **N^{tz}ADPH** from 0 (cyan) to 180 minutes (green) and final oxidative regeneration of **N^{tz}ADP$^+$** by GR (grey and black). c) Normalized emission area over time for the conversion of **N^{tz}AD$^+$** to **N^{tz}ADPH** (blue, λ_{ex}: 330 nm) and NAD$^+$ to NADPH (black, λ_{ex}: 335 nm). d) HPLC traces monitored at 330 nm for the enzymatic conversion of **N^{tz}AD$^+$** (red) to **N^{tz}ADPH** (blue), before the addition of NADK, after 180 min and after GR-mediated oxidation of **N^{tz}ADPH** to **N^{tz}ADP$^+$** (cyan).

In this study we demonstrated that **N^{tz}AD$^+$** could be enzymatically prepared from ^{tz}**ATP** in a considerably higher yield when compared to classical synthetic pathways.[23] The nicotineamide-containing emissive cofactor can then be enzymatically converted to **N^{tz}ADP$^+$**, which, similarly to **N^{tz}AD$^+$**, displays responsive photophysical features, allowing one to monitor enzymatic processes in real-time by visible emission spectroscopy. We then illustrated that those synthetic emissive analogues could replace the native cofactors in complex enzymatic networks. Furthermore, we finally showed that **N^{tz}ADPH**, the enzymatically generated reduced form, can be converted back to **N^{tz}ADP$^+$** using judiciously selected enzymatic reactions. Interestingly, this **N^{tz}ADP$^+$/N^{tz}ADPH** couple showed a photophysical behavior complementary to the photophysical changes seen for the native NADP$^+$ and NADPH.

Our observations suggest that the ^{tz}**A**-based analogues are faithful surrogates of the corresponding native adenosine-based cofactors. Of particular significance is the ability to perform such enzyme-mediated reactions in a sequential manner, exploiting in situ generated substrates and cofactors. Coupled to their useful photophysical characteristics, such synthetic

responsive cofactors can find utility as tools for monitoring redox reactions in *in vitro* biophysical and discovery assays or potentially in living systems.

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Keywords: Cofactors • Kinase • NAD^+ • $NADP^+$ • Nucleotides

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